DNA methylation in rat stomach and duodenum following chronic exposure to N-methyl-N -nitro-N-nitrosoguanidine and the effect of dietary taurocholate

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Abstract: N-Methyl-N -nitro-N-nitrosoguanidine (MNNG) induces a high incidence of carcinomas in the glandular stomach of rats following chronic administration in the drinking water. We determined the level of 7-methylguanine and O6-mehtylguanine in gastric and duodenal DNA during chronic exposure to MNNG (80 p.p.m.). After considerable fluctuations during the initial 3 weeks, levels of methylpurines reached a steady state which was approximately three times higher in the pylorus (i.e. the preferential site of tumor induction)than in the fundus and duodenum, with 7-methylguanine and O6-methylguanine values in the rangeof 520 and 110 mol/mol guanine, respectively. When rats were given MNNG in the drinking water at concentrations ranging from 10 to 80 p.p.m. for 3 weeks, levels of methylpurines reached maximum values already at 10-20 p.p.m. At higher MNNG concentrations, there was no further increase in DNA alkylation. The reason for this lack of dose response remained unclear. Immunohistochemical analyses showed that DNA methylatlon by MNNG is restricted to epithelial cells bordering the luminal surface. The possibility exists that in this target cell population the content of free thiols is a limiting factor for the decomposition of MNNG and its reaction with macromolecules in the gastric mucosa. Addition to the diet of sodium taurocholate, a bile acid previously shown to enhance MNNG-induced stomach carcinogenesis, did not influence the extent of DNA methylation, indicating that it acts as a promoter

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DNA methylation in rat stomach and duodenum following chronic exposure to *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine and the effect of dietary taurocholate

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***N*-Methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) induces a high incidence of carcinomas in the glandular stomach of rats following chronic administration in the drinking water. We determined the level of 7-methylguanine and O^6-methylguanine in gastric and duodenal DNA during chronic exposure to MNNG (80 p.p.m.). After considerable fluctuations during the initial 3 weeks, levels of methylpurines reached a steady state which was approximately three times higher in the pylorus (i.e. the preferential site of tumor induction) than in the fundus and duodenum, with 7-methylguanine and O^6-methylguanine values in the range of 520 and 110 μmol/mol guanine, respectively. When rats were given MNNG in the drinking water at concentrations ranging from 10 to 80 p.p.m. for 3 weeks, levels of methylpurines reached maximum values already at 10–20 p.p.m. At higher MNNG concentrations, there was no further increase in DNA alkylation. The reason for this lack of dose response remained unclear. Immunohistochemical analyses showed that DNA methylation by MNNG is restricted to epithelial cells bordering the luminal surface. The possibility exists that in this target cell population the content of free thiols is a limiting factor for the decomposition of MNNG and its reaction with macromolecules in the gastric mucosa. Addition to the diet of sodium taurocholate, a bile acid previously shown to enhance MNNG-induced stomach carcinogenesis, did not influence the extent of DNA methylation, indicating that it acts as a promoter.**

Introduction

Oral administration of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG*) selectively induces stomach carcinomas in a variety of experimental animals (1–3). The sequential development of MNNG-induced carcinomas in rats has been extensively investigated (4). Most authors agree that histopathologically these neoplasms closely resemble human stomach cancer. We have previously shown that the organ-specific carcinogenicity of MNNG is based on a high extent of DNA methylation in the target tissue. Following a single oral dose (2.5 mg/kg), 7-methylguanine and O^6-methylguanine concentrations in the glandular stomach were nine and 20 times higher than in DNA of forestomach and esophagus, respectively (5). This study also indicated that the interaction of MNNG with DNA of the target organ is due to high concentrations in the gastric mucosa of cellular thiols (e.g. cysteine, reduced glutathione) which greatly accelerate the non-enzymic decomposition of MNNG.

*Abbreviations: MNNG, *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine

The present study was undertaken to determine the extent of DNA methylation during chronic exposure to MNNG. Biochemical analyses were carried out in pylorus and fundus, i.e. stomach regions differing in their susceptibility to MNNG-induced carcinogenesis (2). In addition, we investigated the effect of dietary sodium taurocholate which has previously been shown to markedly increase the incidence of stomach carcinomas (6). The mechanism of this enhancing effect remained unclear. Although a promoting effect is most likely, we felt that the possibility should be ruled out that taurocholate interferes with the breakdown of MNNG and/or its interaction with macromolecules in the gastric mucosa.

Materials and methods

Animals

Experiments were carried out with young male Wistar rats (150–200 g body weight). This inbred strain has been kept for over 50 years in the Institute of Experimental Gerontology in Basel (Switzerland) and since 1961 at the Institute of Pathology, University of Bonn (FRG) from where they were generously donated to the University of Tokyo. This strain has previously been used in histopathological studies on the development of MNNG-induced stomach cancer in rats (4).

Chemicals

MNNG was purchased from Aldrich Chemicals Co., Milwaukee (WI, USA). For experimental use, it was dissolved in distilled water (final concentration, 10, 20, 40 and 80 p.p.m.). The drinking solution was changed every 3 days, MNNG concentrations were checked by HPLC using RP-18 columns (Shandon ODS Hypersil, 4.6 × 250 mm) eluted with 45% (v/v) aqueous ethanol. The results obtained showed a 30% loss within 3 days, corresponding to a half life of ~5 days. This decrease in MNNG concentration is similar to that observed by Sugimura *et al.* (7). Sodium taurocholate was purchased from Wako Pure Chemical Co., Tokyo.

Animal experiments

Animals were divided into four experimental groups. Group 1 (56 rats) was subdivided into seven groups (eight animals each) which were exposed to MNNG (80 p.p.m.) in the drinking water for 3, 6, 9, 14, 21, 42 and 84 days. Group 2 (56 animals) was similarly subdivided and treated but was given a standard laboratory diet which in contrast to group 1 contained 0.25% (w/w) sodium taurocholate. Group 3 consisted of 8 control rats which were kept for 20 days on standard diet and tap water *ad libitum*. Animals of group 4 (40 rats) received MNNG in the drinking water at concentrations ranging from 10 to 80 p.p.m. over a period of 21 days. Animals were allowed to drink *ad libitum*. No attempt was made to assess the precise volume of water intake but there was no indication of significant group-to-group variations.

DNA isolation and analysis

Tissues were rapidly removed and briefly rinsed with saline. Stomach fundus, pylorus and duodenum were separated and frozen in liquid N_2. DNA was isolated from the pooled tissues of eight rats by phenol extraction and adsorption onto hydroxylapatite as previously described (8). Following mild acid hydrolysis (0.1 M HCl at 37°C for 20 h), the amounts of 7-methylguanine and O^6-methylguanine were determined by HPLC using a modification (9) of the procedure of Swenberg and Bedell (10). Briefly, purine bases were separated on a strong cation exchange column (Partisil SCX, 0.46 × 250 mm), eluted at 2 ml/min with 50 mM $NH_4H_2PO_4$, at pH 2 (7-methylguanine), or with the same buffer containing 10% (vol/vol) methanol (O^6-methylguanine). Under these conditions, 7-methylguanine eluted at 9.5 min and O^6-methylguanine at 8.7 min. Quantitation of methylpurines was carried out with Shimadzu spectrofluorophotometer (RF-540), set at 295 nm for excitation and 370 nm for emission. Calibration of the fluorescence signal was performed by injecting radiolabelled methylpurines and determining both radioactivity and fluorescence.

Immunohistochemistry

The organs were removed rapidly and quickly frozen onto small aluminium plates placed directly on slabs of dry ice. Characteristics of the rabbit antiserum (NPZ 193-1) raised against keyhole limpet hemocyanin conjugates of O^6-methylguanosine have been described earlier (11). Briefly, we found 3-fold lower reactivity with O^6-ethyldeoxyguanosine and no cross reactivity with O^6-hydroxyethyldeoxyguanosine. Neither adduct has been shown to result from the reaction of MNNG metabolites with cellular DNA. The antiserum was used without prior absorption. The procedure of Heyting *et al.* (12) and Menkveld *et al.* (13) was used with several modifications, generously communicated by Dr E.Scherer and colleagues at The Netherlands Cancer Institute, Amsterdam. Cryostat sections (6–10 μm) were mounted on ovalbumin-coated slides. Endogenous peroxidase was inactivated by a 45 min incubation with 0.3% H_2O_2 in methanol. After rehydration via graded ethanol, sections were equilibrated with 10 mM EDTA and 10 mM Tris, pH 8.0, for 5 min and subsequently treated for 60 min at 37°C with RNase A (200 μg/ml) and RNase T_1 (50 U/ml) in the same buffer. The sections were then rinsed with distilled water and fixed for 1 min with 40% ethanol, treated for 10 min at room temperature with 50 mM NaOH in 40% ethanol to denature the DNA, neutralized with 5% glacial acetic acid in 40% ethanol, rinsed once with water, incubated for 5 min in wash buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.25% gelatine, 0.05% Triton X-100), and then rinsed in phosphate-buffered saline (PBS, 123 mM NaCl, 8.3 mM Na_2HPO_4, 3.2 mM KH_2PO_4, pH 7.4).The sections were subsequently pre-incubated (60 min, 37°C) with antibody dilution buffer (10% heat-inactivated non-immune swine serum in PBS containing 0.04% Triton X-100). The reaction with the anti-O^6-methyldeoxyguanosine serum (diluted 1:5000 or 1:10 000) was carried out for 16 h at 4°C. After this and all following incubation steps, the sections were washed once with PBS, once with wash buffer and again with PBS. Bound antibodies were detected by the 'double PAP' staining procedure (14), which involved successive incubations with swine anti-rabbit Ig, peroxidase-(rabbit)antiperoxidase complex, swine anti-rabbit Ig, and peroxidase-(rabbit)antiperoxidase complex, each carried out for 45 min at room temperature. Enzymatic activity was visualized by incubation in 50 mM Tris–HCl, pH 7.4, 3,3-diaminobenzidine 4 HCl (0.5 mg/ml) and 0.015% H_2O_2 for 5–10 min at room temperature. Before further processing (dehydration and mounting), the sections were washed with distilled water.

Results

Concentrations of alkylpurines in gastric and duodenal DNA during chronic administration of MNNG (80 p.p.m.) in the drinking water are shown in Figure 1. In all tissues examined, levels of 7-methylguanine and O^6-methylguanine showed considerable variation during the first three weeks of exposure. Later on, a steady state was reached which in the pylorus amounted to ~520 μmol 7-methylguanine and 110 μmol O^6-methylguanine/mol guanine. These values were three times higher than those of fundus and duodenum which themselves differed little from each other. Addition of sodium taurocholate to the standard laboratory diet (0.25%) did not significantly affect the extent of DNA methylation.

In a second experiment animals were given various concentrations of MNNG in the drinking water (10, 20, 40, 80 p.p.m.) and killed after 21 days of exposure. The data obtained (Figure 2) clearly indicate that MNNG concentrations higher than 10–20 p.p.m. do not lead to a proportional increase in the formation of 7-methylguanine. O^6-Methylguanine levels showed a similar lack of dose response. The amounts determined at 20 and 80 p.p.m. were 84 and 74 (pylorus), 21 and 24 (fundus), and 23 and 26 (duodenum) μmol/mol, respectively. Immunohistochemical studies were carried out in animals exposed to MNNG (40 or 80 p.p.m.) for 3 weeks. They showed that DNA methylation is restricted to cells located near the gastric lumen. Again, there was evidence of a regionally different extent of alkylation. In the pyloric mucosa, immunoreactivity generally yielded a stronger signal and O^6-methylation of nuclear DNA could easily be identified at levels down to 10 epithelial cells below the luminal surface (Figure 3). In the fundus region, unequivocal immunoreactivity to O^6-methyldeoxyguanosine was usually restricted to the cells directly bordering the gastric lumen. No stained nuclei were detected in sections from untreated rats (not shown).

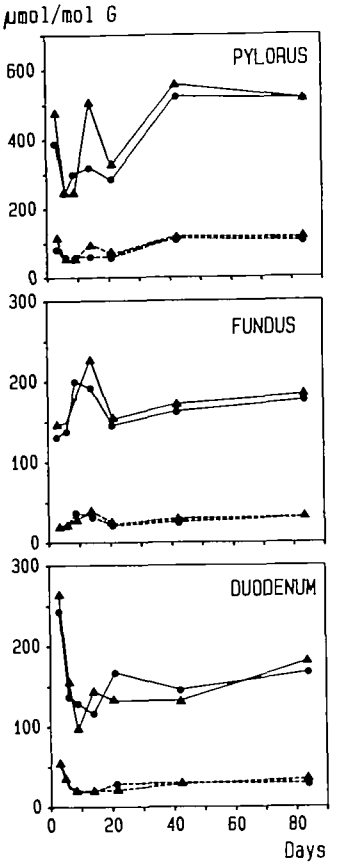

Fig. 1. Concentrations of 7-methylguanine (solid line) and O^6-methylguanine (dotted line) in DNA of stomach (pylorus, fundus) and duodenum during chronic exposure to MNNG in the drinking water (80 p.p.m.) over a period of 3–84 days One group of rats was treated with MNNG alone (●); the other group received MNNG plus a diet containing 0.25% (w/w) sodium taurocholate (▲).

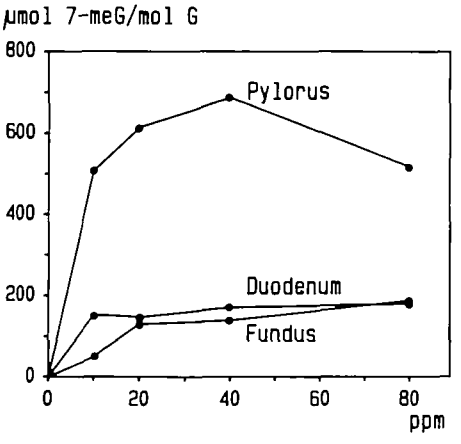

Fig. 2. Levels of 7-methylguanine in DNA from pylorus, fundus and duodenum after 21 days of exposure of MNNG in the drinking water at concentrations ranging from 10–80 p.p.m.

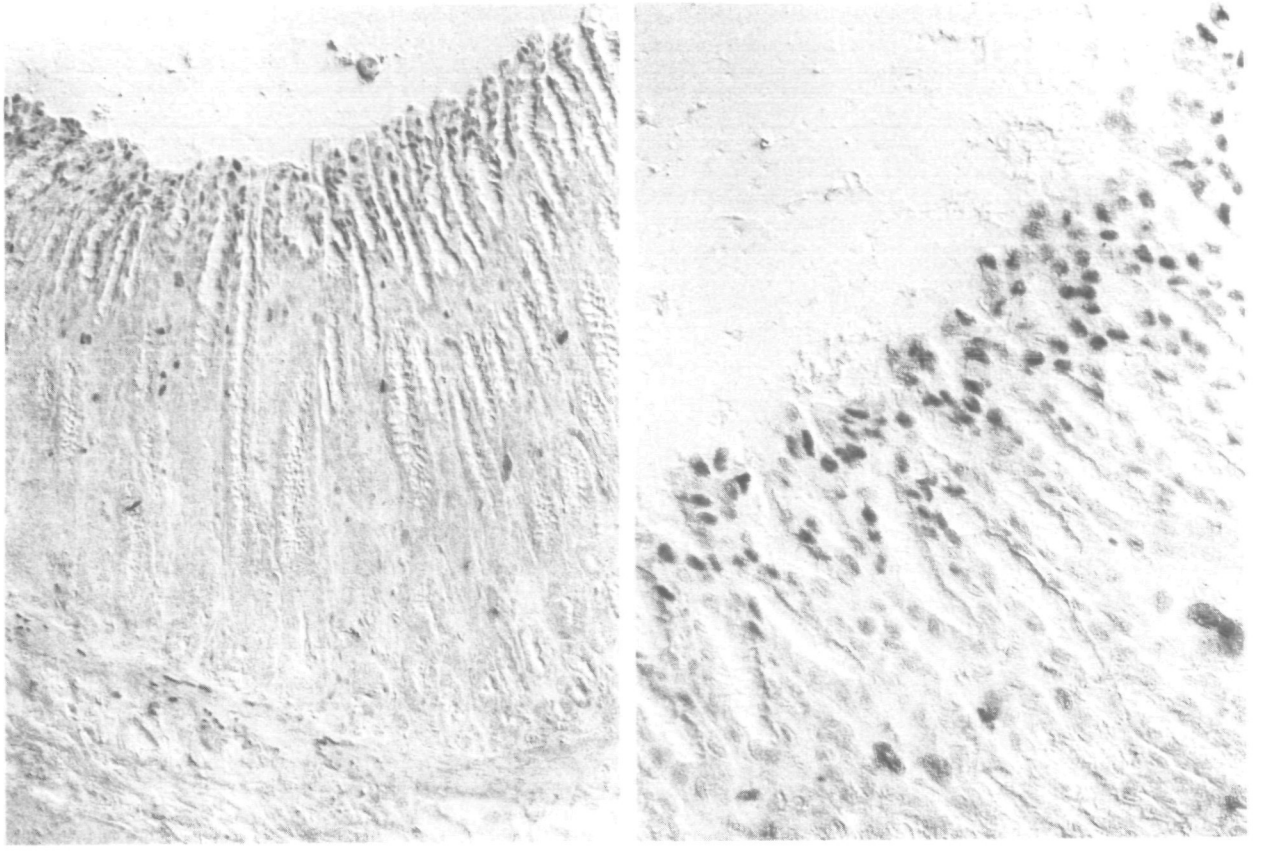

Fig. 3. Immunohistochemical demonstration of O^6-methyldeoxyguanosine in pyloric cell nuclei of rats which received MNNG in the drinking water (80 p.p.m.) over a period of 3 weeks. Note that immunoreactivity is restricted to a small layer of cells bordering the luminal surface. Nomarski interference contrast microscopy (×40 and ×80).

Discussion

The objective of the present study was to determine levels of methylpurines in stomach and duodenum of rats chronically exposed to MNNG in the drinking water. Since epithelia of these tissues have a high rate of cell turnover, we expected that steady state levels be reached within 3–6 days after the onset of MNNG administration. High concentrations were indeed observed within three days of exposure (Figure 1). However, values varied considerably during the initial stage of the experiment. The most likely explanation for this phenomenon is that MNNG causes stomach toxicity with erosions and subsequent cellular repair and thus larger group-to-group variations in the level of DNA alkylation than observed at later stages of continuous MNNG administration. Histopathological analyses have shown that these lesions occur most frequently during the first 1–2 weeks of exposure (15). After ~3 weeks, levels of DNA methylation reached a steady state and this probably reflects an adaptation of the gastric mucosa to the toxic effects of MNNG. The lack of a dose response during chronic exposure to MNNG concentrations beyond 10–20 p.p.m. was unexpected since this carcinogen does not require enzymic bioactivation and would thus be expected to cause DNA alkylation proportional to dose. We have considered the possibility that voluntary water intake is reduced at high MNNG concentrations. Measurements showed considerable day-to-day fluctuations. Although there was a tendency towards reduced uptake at high MNNG concentrations, these differences were too small to account for the observed lack of dose response. Since thiols markedly enhance the decomposition of MNNG, their presence at high concentrations in the gastric mucosa has been linked to the organ-specific carcinogenicity of MNNG (5). Our immunohistochemical studies showed that the reaction of MNNG with target DNA is restricted to a small number of epithelial cells bordering the gastric lumen. A similar distribution was observed autoradiographically following a single dose of [^{3}H-methyl]MNNG (16). The possibility, therefore, exists that free thiols in pyloric epithelia are easily consumed in this reaction and thereby limit the extent of DNA alkylation. Attempts to histochemically demonstrate an MNNG-induced depletion of free thiols in the superficial layers of the gastric mucosa failed although this effect could easily be shown after intragastric administration of the thiol-blocking agent, *N*-ethylmaleimide (unpublished results).

Since O^6-methylguanine and related *O*-alkylated bases represent critical DNA lesions responsible for the initiation of malignant transformation by simple alkylating agents (17), our results would suggest that the carcinogenic efficiency of MNNG in this tumour model is largely independent of the concentration of MNNG in the drinking water. This view is supported by a report by Sugimura *et al.* (18) in which rats were chronically exposed to MNNG at levels of 33 or 83 μg/ml in the drinking water. This considerable difference in dose was not paralleled by either differences in the incidence of stomach carcinomas or the time interval at which 50% of rats had died from tumours (~330 and 380 days).

When MNNG and related methylating nitroso compounds react with DNA *in vitro*, i.e. in the absence of repair enzymes, the ratio of the amounts of O^6-methylguanine and 7-methylguanine

formed is 0.11 (17). In the present study we observed a consistently higher ratio of ~0.16 (Figure 1). Since the loss of methylpurines due to mucosal cell turnover is similar for both adducts, this finding indicates that removal of 7-methylguanine by spontaneous depurination and glycosylase-mediated excision repair (19) is somewhat faster than the repair of O^6-methylguanine by the O^6-alkylguanine-DNA alkyltransferase. During the course of the experiment, the ratio remained stable, suggesting that in contrast to rat liver (20) carcinogen-induced toxicity does not lead to an induction of the alkyltransferase in the stomach mucosa.

In a previous study (5), we reported that DNA methylation by MNNG in the glandular stomach is nine times higher than in the forestomach and 20 times higher than in the esophagus. The present study provides evidence that within the target tissue, too, the extent of DNA alkylation closely correlates with the site of tumour induction. Morphological analyses have shown that after oral administration in the drinking water MNNG-induced adenocarcinomas are selectively located in the gastric pylorus (2), in particular at the lesser curvature (21). We found that levels of 7-methylguanine and O^6-methylguanine in this region were 3-fold higher than in DNA of the gastric fundus and the duodenum and this was also apparent on immunohistochemical stains for O^6-methylguanine (not shown). This phenomenon, too, remains unexplained. Thiol levels are known to be considerably higher in the glandular stomach than in the squamous epithelium of the forestomach (5) but no data are available on the concentration of free thiols in different regions of the glandular stomach. One may also argue that the bulk of fluid volume consumed runs along the small curvature and that a homogeneous distribution within the stomach lumen does not occur under physiological conditions. In contrast, MNNG administration by gavage produces carcinomas in various stomach regions including the forestomach (22) and autoradiographic studies have shown that following [^{14}C]MNNG application by stomach tube, ^{14}C-labelled reaction products are distributed over the entire stomach wall, rather than being concentrated in the pyloric region (5).

It has recently been reported that bile acid (taurocholic acid) greatly enhances MNNG-induced stomach tumorigenesis (6,23,24). In these studies, even small amounts of MNNG induced a high incidence of stomach tumours when taurocholic acid was simultaneously or subsequently added to the diet or in the drinking water, thus indicating a promoting effect. The present study supports this view. Co-administration of taurocholate did not affect the extent of interaction of MNNG with DNA in the target tissue.

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References

1. Kogure,K., Sasadaira,H., Kawachi,T., Shimosato,Y., Tokunaga,A., Fujimura,S. and Sugimura,T. (1974) Further studies on induction of stomach cancer in hamsters by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Br. J. Cancer*, **29**, 132–142.
2. Sugimura,T. and Kawachi,T. (1978) Experimental carcinogenesis. In Lipkin,M. and Good,R.A. (eds), *Gastrointestinal Tract Cancer*, Plenum Publishing Corp., New York, pp. 327–341.
3. Ohgaki,H. and Sugimura,T. (1988) Experimental stomach cancer. In Douglass,Jr.,H.O. (ed.), *Gastric Cancer*, Churchill Livingstone, New York, Edinburgh, London, Melbourne, pp. 27–54.
4. Kobori,O., Gedigk,P. and Totovic,V. (1976) Early changes of glandular stomach in Wistar rats ingesting *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG). *Z. Krebsforsch.*, **87**, 127–138.
5. Wiestler,O., von Deimling,A., Kobori,O. and Kleihues,P. (1983) Location of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-induced gastrointestinal tumors correlates with thiol distribution. *Carcinogenesis*, **4**, 879–883.
6. Kobori,O., Shimizu,T., Maeda,M., Atomi,Y., Watanabe,J., Shoji,M. and Morioka,Y. (1984) Enhancing effect of bile and bile acid on stomach tumorigenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *J. Natl. Cancer Inst.*, **73**, 853–861.
7. Sugimura,T., Fujimura,S., Kogure,K., Baba,T., Saito,T., Nagao,M., Hosoi,H., Shimosato,Y. and Yokoshima,T. (1969) Production of adenocarcinomas in glandular stomach of experimental animals by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Gann Monogr. Cancer Res.*, **8**, 157–196.
8. von Hofe,E., Grahmann,F., Keefer,L.K., Lijinsky,W., Nelson,V. and Kleihues,P. (1986) Methylation *versus* ethylation of DNA in target and non-target tissues of Fischer 344 rats treated with *N*-nitrosomethylethylamine. *Cancer Res.*, **46**, 1038–1042.
9. von Hofe,E. and Kleihues,P. (1986) Comparative studies on hepatic DNA alkylation in rats by *N*-nitrosomethylethylamine and *N*-nitrosodimethylamine plus *N*-nitrosodiethylamine. *J. Cancer Res. Clin. Oncol.*, **112**, 205–209.
10. Swenberg,J,A. and Bedell,M.A. (1982) Cell-specific DNA alkylation and repair: Application of new fluorometric techniques to detect adducts. In Magee,P.N. (ed.), *Banbury Report 13: Indicators of Genotoxic Exposure*. Cold Spring Harbor Laboratory, p. 205–220.
11. Ludeke,B.I. and Kleihues,P. (1988) Formation and persistence of O^6-(2-hydroxyethyl)-2'-deoxyguanosine in DNA of various rat tissues following a single dose of *N*-nitroso-*N*-(2-hydroxyethyl)urea. An immuno-slot-blot study. *Carcinogenesis*, **9**, 147–151.
12. Heyting,C., Van Der Laken,C.J., Van Raamsdonk,W. and Pool,C.W. (1983) Immunohistochemical detection of O^6-ethyldeoxyguanosine in the rat brain after *in vivo* applications of *N*-ethyl-*N*-nitrosourea. *Cancer Res.*, **43**, 2935–2941.
13. Menkveld,G.J., Van Der Laken,C.J., Hermsen,T., Kriek,E., Scherer,E. and Den Engelse,L. (1985) Immunohistochemical localization of O^6-ethyldeoxyguanosine and deoxyguanosin-8-yl-(acetyl)aminofluorene in liver sections of rats treated with diethylnitrosamine, ethylnitrosourea or *N*-acetylaminofluorene. *Carcinogenesis*, **6**, 263–270.
14. Ordronneau,P., Lindström,P.B.M. and Petrusz,P. (1981) Four unlabeled antibody bridge techniques: a comparison. *J. Histochem. Cytochem.*, **29**, 1397–1404.
15. Ohgaki,H., Kusama,K., Hasegawa,H., Sato,S., Takayama,S. and Sugimura,T. (1986) Sequential histologic changes during gastric carcinogenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in susceptible ACI and resistant Buffalo rats. *J. Natl. Cancer Inst.*, **77**, 747–755.
16. Ohgaki,H., Tomihari,M., Sato,S., Kleihues,P. and Sugimura,T. (1988) Differential proliferative response of gastric mucosa during carcinogenesis induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in susceptible ACI rats, resistant Buffalo rats, and their hybrid F1 cross. *Cancer Res.*, **48**, 5275–5279.
17. Singer,B. and Grunberger,D. (1983) *Molecular Biology of Mutagens and Carcinogens*, Plenum Press, New York and London.
18. Sugimura,T., Fujimura,S. and Baba,T. (1970) Tumor production in the glandular stomach and alimentary tract of the rat by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Cancer Res.*, **30**, 455–465.
19. Margison,G.P. and Pegg,A.E. (1981) Enzymatic release of 7-methylguanine from methylated DNA by rodent liver extracts. *Proc. Natl. Acad. Sci. USA*, **78**, 861–865.
20. Margison,G.P. (1982) Chronic or acute administration of various dialkylnitrosamines enhances the removal of O^6-methylguanine from rat liver DNA *in vivo*. *Chem.-Biol. Interactions*, **38**, 189–201.
21. Kobori,O. (1980) Analytical study of precancerous lesions in rat stomach mucosa induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *Gann Monogr.*, **25**, 141–150.
22. Hirono,I. and Shibuya,C. (1972) Induction of stomach cancer by a single dose of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine through a stomach tube. In Nakahara,W., Takayama,S., Sugimura,T. and Odashima,S. (eds), *Topics in Chemical Carcinogenesis*, University of Tokyo Press, pp. 121–132.
23. Salmon,R.J., Laurent,M. and Thierry,J.P. (1984) Effect of taurocholic acid feeding on methyl-nitro-*N*-nitroso-guanidine induced gastric tumors. *Cancer Lett.*, **22**, 315–320.
24. Cohen,A., Geller,S.A. and Horowtiz,I., Toth,L.S. and Werther,L.J. (1984) Experimental models for gastric leiomyosarcoma. The effects of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in combination with stress, aspirin, or sodium taurocholate. *Cancer*, **53**, 1088–1092.

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